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Abnormal Lipid Metabolism in Tauopathy Brains: Dysregulation and Intervention

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

Yuhang Nie

Committee in charge:

Professor Xu Chen, Chair
Professor Enfu Hui, Co-Chair
Professor Aaron Coleman

2024

The thesis of Yuhang Nie is approved, and it is acceptable in quality and form for the publication on microfilm and electronically.

University of California San Diego

2024

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ABSTRACT OF THE THESIS

Abnormal Lipid Metabolism in Tauopathy Brains: Dysregulation and Intervention

by

Yuhang Nie

Master of Science in Biology

University of California San Diego, 2024

Professor Xu Chen, Chair
Professor Enfu Hui, Co-Chair

Dysregulation of lipid metabolism is implicated in neurodegenerative diseases like Alzheimer's disease (AD). Lipid droplets (LDs) are dynamic organelles essential for cellular lipid metabolism and homeostasis. Investigating the interplay between LDs and AD pathology offers insights into disease mechanisms and therapeutic avenues. Here, we studied LD accumulation in tauopathy brains, utilizing stimulated Raman scattering imaging to visualize LD distribution in fly and mouse models of tauopathy, iPSC-neurons and a microglia cell line. . LD accumulation in the brain was accompanied by increased expression of lipogenesis genes and LD-associated proteins. Remarkably, LD accumulation was predominantly found in activated microglia, suggesting an interplay between tauopathy neurons and microglia. Activation of neuronal AMPK reduced LD accumulation and neuroinflammation *in vitro* and *in vivo*. Our findings elucidate the intricate link between AMPK, LD biology, and tau pathology, offering insights into therapeutic strategies for neurodegenerative diseases. On the other hand, we investigated the effects of β-hydroxybutyrate (BHB), a ketone body produced by fat metabolism implicated in neuroprotection, using a fly model of tauopathy and a microglia cell line BHB supplementation ameliorated tauopathy phenotypes in flies and reduced LD accumulation in microglia, primarily through the signaling activity of BHB. Together, our results suggest that targeting lipid metabolism could be a potential therapeutic intervention for tauopathies and neuroinflammation.

CHAPTER I: INTRODUCTION

Alzheimer's Disease and Tauopathy

Alzheimer's Disease (AD) is one of the most common causes of dementia. The characterization of AD includes impaired cognitive ability, memory loss and declined motor function (Kluger et al., 1997). Currently, more than 6 million people over 65 years old are suffering from AD in the United States and this number is expected to double in the next 30 years. The mechanism of AD pathology remains unclear. AD is characterized by pathological aggregation of Aβ and Tau in the brain, forming Aβ plaques and neurofibrillary tangles (NFT), respectively. Besides AD, a group of neurodegenerative disorders collectively called tauopathies, including Parkinson's Disease (PD), frontotemporal dementia (FTD), corticobasal degeneration (CBD), are characterized by the abnormal aggregation of Tau protein in the brain (Gao et al., 2018). Tauopathies are associated with neuronal dysfunction, synaptic loss, and cognitive impairment, ultimately leading to progressive neurodegeneration. Tau protein normally binds to microtubule in neurons and aids with neuronal structure and axonal transport (Barbier et al., 2019). Under pathological conditions, Tau protein becomes hyperphosphorylated, forming aggregates and eventually NFT in tauopathy brain (Šimić et al., 2016). It's been shown that toxic Tau species can impair the health and function of the brain, causing synaptic dysfunction, neuroinflammation and neurodegeneration. Understanding the mechanism of Tau pathophysiology is crucial for developing effective, targeted therapies that could slow or stop disease progression.

Metabolic change in tauopathy brains

Alterations in glucose metabolism in the brain is a pivotal feature of tauopathies, especially AD. Glucose serves as the primary energy source for the brain, furnishing over 90% of its energy requirements (Sokoloff et al., 1977). However, tauopathy brains exhibit disruptions in glucose utilization, as evidenced by reduced glucose uptake and metabolism in regions vulnerable to Tau pathology, such as the temporal, parietal, and frontal cortices, as observed in positron emission tomography (PET) studies (Mosconi et al., 2009). This regional hypometabolism correlates with areas of heightened Tau deposition (Boccalini et al., 2023), highlighting the intimate association between metabolic dysfunction and Tau-mediated neurodegeneration.

Amidst impaired glucose metabolism, an adaptive response emerges in the form of upregulated lipid metabolism, offering an alternative energy source to sustain neuronal viability in tauopathy brains. Lipids play multifaceted roles in cellular structure, signaling pathways, and energy storage. Dysregulated lipid metabolism in tauopathies leads to alterations in lipid composition and metabolism, as evidenced by studies utilizing metabolomics and lipidomic approaches (Han et al., 2011). Notably, lipid droplet (LD) accumulation has been observed in postmortem AD brains, indicative of aberrant lipid metabolism and storage in disease states (Hamilton et al., 2015).

Although altered lipid metabolism in tauopathy brains may serve as a compensatory mechanism to meet energy demands, it could potentially exacerbate the disease state. Dysregulated lipid metabolism has been implicated in promoting Tau phosphorylation, thereby exacerbating Tau pathology (Laurent et al., 2016). Additionally, dysregulation of lipid

metabolism leads to the production of pro-inflammatory lipid mediators, inducing oxidative stress and fostering a neuroinflammatory milieu that exacerbates neurodegeneration in tauopathy brains (Lukiw, 2012). Furthermore, dysregulated lipid metabolism may exacerbate neuronal dysfunction in tauopathies, as lipids play crucial roles in synaptic membrane fluidity and neurotransmitter release, influencing synaptic transmission and plasticity (Petrov et al., 2019). Understanding the intricate interplay between aberrant lipid metabolism and Tau-mediated neurodegeneration is crucial for deciphering disease mechanisms and developing targeted therapeutic interventions. On the other hand, emerging evidence suggests that targeting metabolic pathways holds promise for mitigating neurodegeneration and halting disease progression in tauopathies (Katsouri et al., 2020). Strategies aimed at restoring metabolic homeostasis, including modulating lipid metabolism, and enhancing energy production, represent potential therapeutic avenues for AD and related tauopathies. For instance, ketogenic diet (KD), which is a diet structure of high fat and low carbohydrate, were served as one of these strategies. KD promotes the usage of lipids as an energy source to compensate for the energy gap caused by glucose hypometabolism. And it's been shown that KD is beneficial for AD patients in terms of promoting cognitive abilities and long-term memory (Rusek et al., 2019). The bidirectional relationship between lipid metabolism and Tau pathophysiology underscores the need for fully understanding the mechanism underlying this dysregulated lipid metabolism to develop comprehensive therapeutic approaches for tauopathies (Boutajangout et al., 2011).

Lipid Droplets

Lipid droplets (LDs) represent highly dynamic and multifunctional organelles found in most eukaryotic cells, serving as hubs for lipid metabolism, storage, and signaling. Comprising a hydrophobic core of neutral lipids, primarily triglycerides and cholesterol esters, surrounded by a phospholipid monolayer with associated proteins, LDs regulate lipid fluxes, maintain cellular lipid homeostasis, and support various cellular functions (Pressly et al., 2022). Their pivotal roles extend beyond mere lipid storage; LDs are implicated in diverse cellular processes, including membrane trafficking, energy metabolism, and even regulation of gene expression (Pennetta et al., 2018). These organelles exhibit remarkable plasticity and adaptability, responding to changes in cellular lipid status and metabolic demands.

The synthesis and turnover of LDs are tightly regulated processes orchestrated by a network of lipids and proteins. Central to LD biogenesis is the coordinated action of enzymes involved in lipid metabolism, such as diacylglycerol acyltransferases (DGATs) and adipose triglyceride lipase (ATGL), which catalyze the esterification of fatty acids into triglycerides and the hydrolysis of triglycerides into free fatty acids, respectively (Chitraju et al., 2017). Additionally, proteins associated with LDs, including perilipins, modulate LD dynamics, lipid trafficking, and interactions with other cellular compartments (Kory et al., 2016). The intricate interplay between LD-associated proteins, lipids, and metabolic pathways ensures the proper formation, growth, and turnover of LDs in response to cellular cues and metabolic demands.

Emerging evidence suggests a significant involvement of LD dysfunction in the pathogenesis of tauopathies. For instance, dysregulation of LD-associated proteins and enzymes involved in lipid metabolism disrupts lipid homeostasis and impairs cellular functions.

(Pressly et al., 2022). Understanding the intricate crosstalk between LDs and Tau pathology could unveil novel therapeutic targets and strategies for intervening in the progression of neurodegenerative diseases.

CHAPTER II. NEURONAL AMPK DYSFUNCTION INDUCES LIPID DROPLET ACCUMULATION IN MICROGLIA

INTRODUCTION

lipid droplets represent dynamic organelles essential for lipid metabolism, storage, and cellular homeostasis (Zadoorian et al. 2023). Their intricate regulation and multifaceted roles extend beyond lipid storage to encompass diverse cellular functions and processes. Dysregulation of LD biology is increasingly recognized as a contributing factor in the pathogenesis of neurodegenerative diseases, including tauopathies such as AD (Zhang et al., 2021; Giovedi et al., 2019). Elucidating the molecular mechanisms underlying the interplay between LDs and tau pathology holds promise for developing targeted therapies aimed at mitigating neurodegeneration and preserving cognitive function in patients afflicted with these devastating disorders.

AMP-activated protein kinase (AMPK) is a kinase that plays a crucial role in eukaryote cells to maintain the cellular energy homeostasis. AMPK can be activated in response to alternation of AMP/ADP to ATP ratio in the cell by phosphorylation at Thr172 (Hardie et al., 2011). Activated AMPK stimulates downstream catabolic activities such as glucose uptake, fatty acid oxidation, and inhibits anabolic activities such as lipogenesis or glycogenesis. By involving in multiple metabolic pathways, AMPK serves as a key regulator in maintaining cell energy status (Cui et al., 2023).

To understand the endogenous LD biology in Tauopathy brains, we utilized stimulated Ramen scattering imaging technique (SRS imaging) to visualize the LD distribution *in vivo* and *in vitro*. SRS imaging is a technique based on the two-photon excitation microscopy that

does not rely on conventional fluorescent markers and antibodies to visualize the specific molecules (Tipping et al., 2016). This label-free property of SRS imaging makes it an ideal tool for studying lipid metabolism and LD related phenotypes. Combining SRS imaging technique with conventional genetic and molecular biology techniques, we investigated how neuronal AMPK pathway regulated LD accumulation phenotype in Tauopathy brains by manipulating AMPK signaling pathway.

MATERIAL AND METHODS

Drosophila Model

Drosophila lines: UAS-AMPK.wt, UAS-AMPK.DN, elav^{C155}-GAL4 and was used in this study and originally obtained from BDRC. Homemade Drosophila line: C155/FM7; UAS-hTau1.13/CyO expressing wildtype human Tau in neuron was also used in this study. They have maintained on the standard diets for several generations and then crossed to produce Tau-overexpression Drosophila.

Starvation lifespan assay

C155/FM7; UAS-hTau1.13 female flies were used to cross with either UAS-AMPK.wt male flies or UAS-AMPK.DN male flies. C155/+; UAS-htau1.13/UAS-AMPK.wt flies were chosen in F1 flies. 40 flies from each sex and genotype were collected and separated into 2 vials. All these flies were fed on the standard diet until Day 5 when they were transferred to 1% agar vial. The number of deaths were counted every 12 hours.

qRT-PCR

RNA extraction from animal tissue or cell culture were extracted by using RNeasy Mini Kit following the manufacturer's protocol (QIAGEN, Cat#74104). 25mg mice brain or 100 drosophila dissected brain were used to extract RNA. DNase I digestion were performed to eliminate gDNA contamination. 500ug RNA were used in reverse transcription reaction protocol with iScript Reverse Transcription Supermix for RT-PCR (BIO-RAD, Cat#1708841). 1ul of cDNA were mixed with primers and Maxima STBYGreen qPCR Master Mix (Thermo Fisher, Cat#K0253) and processed qRT-PCR reaction by using CFX96 Real-Time PCR Detection System (BIO-RAD). The relative expression level of interested gene were normalized to housekeeping gene (ACTB) and relative expression level fold change were calculated by $2^{\wedge(-\Delta\Delta Ct)}$ method.

IHC

Mouse brain slices were fixed in 4%PFA for 24h at 4C degree and then transfer to 1xPBS for 30min to wash off the rest of PFA. Brain slices were put in the well of 24 well plate and add 500ul 0.1%PBS-T solution for 30mins. Then, blocking with 500ul 0.02% PBS-T with 5% Normal Goat Serum (NGS) for 1h. Primary antibodies were diluted in 0.02%PBS-T with 2.5% NGS as desired concentration. Brain slices were incubated with primary antibody for 24 hours at 4°C. 3 times of 500 ul 0.02% PBS-T wash was performed to wash off the remaining primary antibody. Then, brain slices were incubated with secondary antibody at a dilution of 1:600 in 0.02% PBS-T with 2.5% NGS at room temperature for 1 hour. 3 times of 500 ul 0.02% PBS-T wash was performed to wash off the remaining secondary antibody. Then, brain slices were

transferred to 1x PBS and stored at 4℃for imaging purposes.

ICC

Coverslips with seeded cells were fixed in 4% PFA for 30 minutes at room temperature and then transferred to 1 x PBS to wash off the rest PFA. Then, cells were treated with 200 ul 0.1% PBS-T solution for 30 minutes and followed by blocking in 200 ul 0.02%PBS-T with 2% Bovine serum albumin (BSA) for 1 hour. Primary antibodies were diluted in 0.02% PBS-T with 2% BSA as desired dilution and incubated with the cells overnight at 4℃. 3 times of 0.02% PBS-T wash were performed to wash of the abundant primary antibody. Then, the cells were incubated with secondary antibody that diluted in 0.02% PBS-T with 2% BSA at a dilution of 1:600 for 1 hour at room temperature. 3 times of 0.02% PBS-T wash were performed to wash of the abundant secondary antibody. Then, the cells were transferred to 500 ul 1 x PBS at 4℃ for imaging purposes.

Western Blot

Fly brain tissue or cell culture samples were lysed in RIPA buffer supplemented with protease/phosphatase inhibitor cocktails (Sigma) and centrifuged at 16,000 x g at 4℃for 15 minutes. Supernatants were collected and protein concentrations were determined by BCA Assay (ThermoScientific). Equal amount of protein was mixed with 4x Buffer Loading Dye (ThermoScientific) and heated at 95℃ for 5 minutes on metal bath. Gel-electrophoresis were performed with 4-12% SDS-PAGE gel (BIO-RAD), and then transferred to nitrocellulase membrane for antibody probing. Bands were detected by an enhanced chemiluminescence kit

(ECL) (ThermoScientific) and visualized using a ChemiDocMP Imaging System (BIO-RAD).

RESULTS

LD accumulation in Tauopathy Drosophila and PS19 mice.

SRS imaging techniques were employed to visualize brain lipid distribution, revealing the accumulation of lipid droplets (LDs) in tauopathy models. In Tau-transgenic Drosophila and PS19 mice, LD accumulation was observed compared to wild-type counterparts, particularly evident in the CA1 region of PS19 mice hippocampus (Fig. 1A, C). Additionally, severe neuronal layer loss was noted in the CA1 region of PS19 mice, correlating significantly with LD numbers in both CA1 and DG regions (Fig. 1B), highlighting LD accumulation as a disease-associated marker.

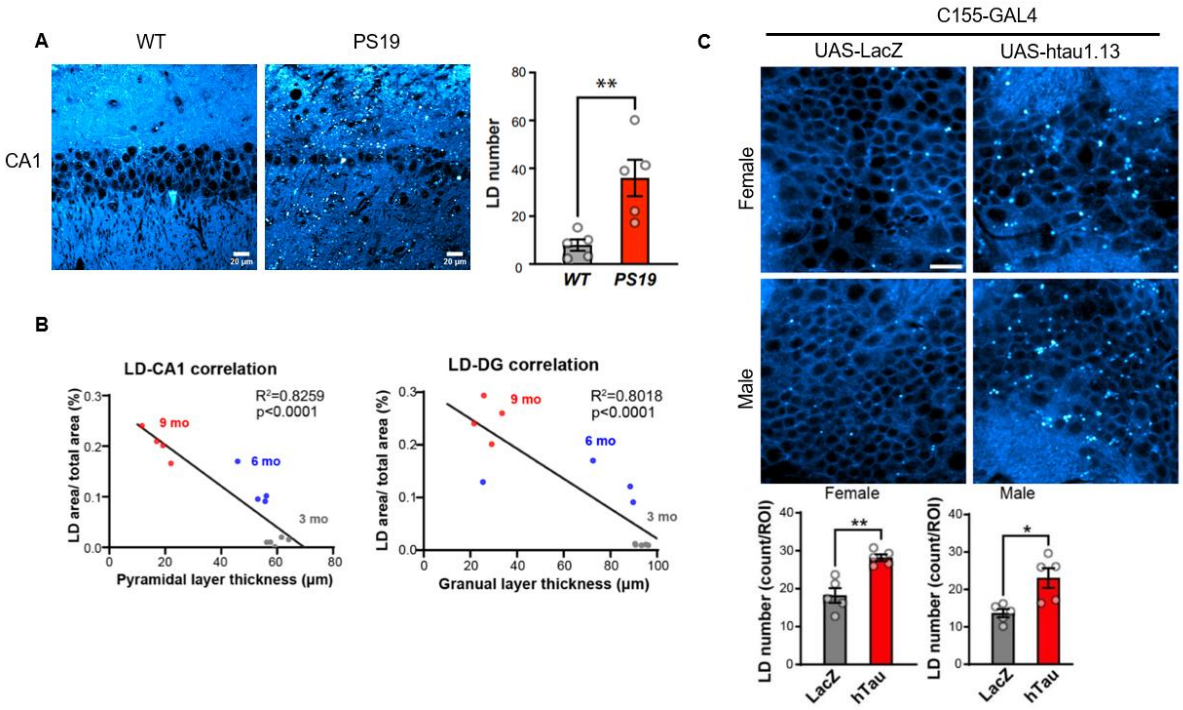


Figure 1. Lipid droplets (LDs) accumulated in tauopathy brains and correlated with neuronal loss level.

(A) SRS images showing endogenous lipid distribution in the CA1 region in hippocampus of wild-type (WT) or tauopathy mice (PS19) at 9 mo.

(B) Pearson analysis showing a negative linear correlation between brain LD abundance and the thickness of CA1 pyramidal layer and DG granule cellular layer in PS19 mice of different ages. Red dots: 9 month old (n=4); blue dots: 6 month old (n=4); grey dots: 3 month old (n=4).

(C) SRS images showing LDs accumulated in 5-day-old female and male hTau flies brain with quantifications.

LD co-localized in microglia cells in PS19 mice

Co-registering of SRS-lipid signal and Immunohistochemistry (IHC) staining for different cell type markers demonstrated colocalization of LD in activated microglia (Fig.2A-i), astrocytes (Fig.2A-ii), oligodendrocytes (Fig.2A-iii) and neurons (Fig.2A-iv) in hippocampus region of PS19 mice brain. These results showed that about 70.1% of LDs were localized in microglia cells in 9-month-old PS19 mice brain (Fig. 2B). More specifically, CD68 staining shows that most of these LD was localized in activated microglia (Fig.2A-i). These results were consistent with what we discovered in the Drosophila brain that most lipid droplets

were localized in the glial cells in the Drosophila brain (Fig.1C).

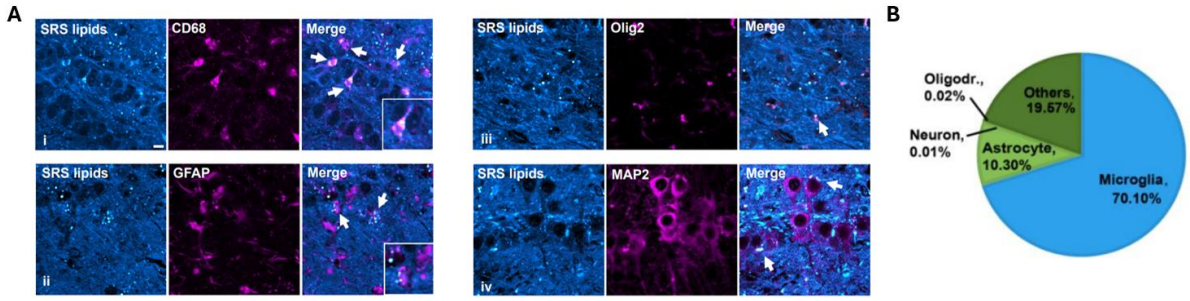


Figure 2. LD mostly colocalized in activated microglia in PS19 mice brain

(A) immunostaining co-registered with SRS imaging showing LDs colocalization with glia- and neuron-specific markers CD68 (i), GFAP (ii), Olig2 (iii), and MAP2 (iv) in PS19 hippocampus.

(B) Percentage distribution of LDs colocalization in different cell types.

Genes involved in LD metabolism were significantly upregulated in Tauopathy models.

To investigate the mechanism for LD accumulation, we used qRT-PCR to test the expression level of lipid metabolism pathway genes and ketone body metabolism pathway genes. Results demonstrated that a significant and consistent upregulation in lipogenesis pathway genes (Fig.3A-B). This result was consistent with what we found in previous RNA-seq result. To validate our finding in the change of expression level of *Plin2/3* gene and correlate this change with LD accumulation in Tauopathy, we next stained Plin3 in mice brain. Results show that Plin3 protein was upregulated in 9-month-old PS19 mice and co-localized with SRS-LD signal (Fig.3C).

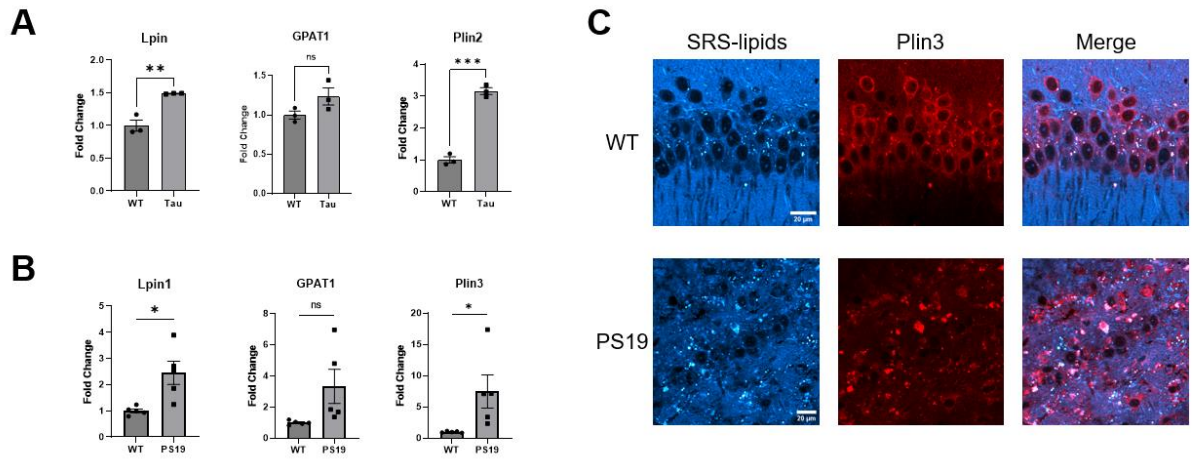


Figure 3. Lipid metabolism pathway gene upregulated in tauopathy brains.

(A) qRT-PCR results showing mRNA levels of *Lpin*, *GPAT1* and *Plin2* (*Lsd-2*) in the brain of hTau flies compared to WT flies. 100 flies per condition were used in this experiment and each data point represents a technical replicate.

(B) qRT-PCR results showing mRNA levels of *Lpin1*, *GPAT1* and *Plin3* in the hippocampus of PS19 mice compared to WT littermates. n=5 per group.

(C) Immunostaining results showing Plin3 colocalization with SRS-LD.

Lipid droplets accumulated in Tau^{V337M} mutant iPSC derived neurons.

It's interesting and surprising that LD accumulated in microglia cells while Tau pathology begins in neurons. We next asked if neurons could accumulate LDs itself. Induced pluripotency stem cells (iPSC) were used to study the LD accumulation in neurons. Wildtype (WTC11) iPSC derived neuron (iPSC-neurons) and Tau^{V337M} homozygous mutant iPSC-neurons were imaged by SRS-imaging technique. LDs accumulated in Tau^{V337M} iPSC-neurons (Fig.4A). Since Lpin1, GPAT1 and Plin2/3 were significantly upregulated in both Tau-transgenic-Drosophila brain and PS19 mice hippocampus region, we next utilized qRT-PCR to measure the mRNA expression level of these three gene in iPSC- neurons to confirm with the SRS-imaging results. qRT-PCR results show that all these three genes were upregulated in Tau^{V337M} homozygous mutant iPSC-neurons (Fig. 4B).

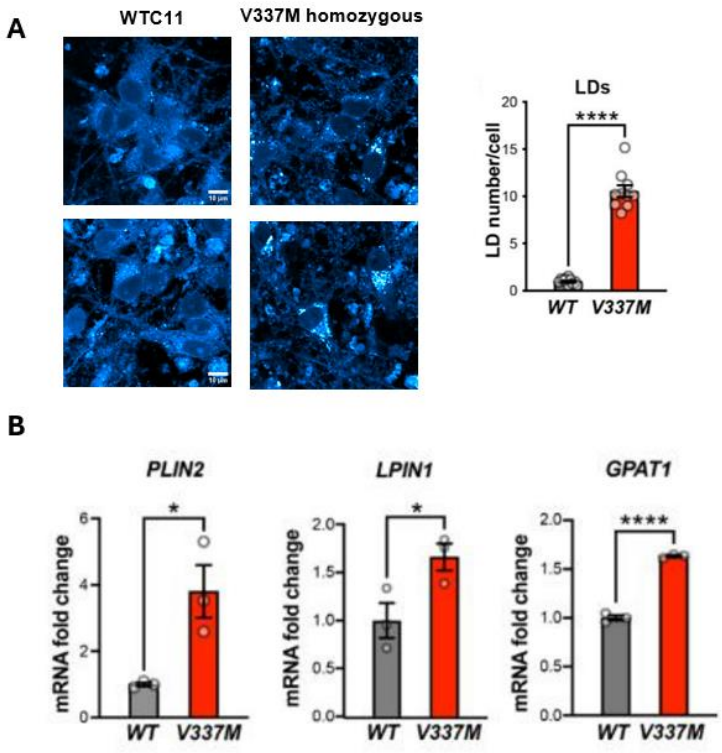


Figure 4. LDs accumulated in Tau mutant iPSC-neurons with upregulation in lipid metabolism pathway genes.

(A) SRS images showing LDs accumulated in Tau mutant iPSC-neurons compared to WT iPSC-neurons with quantification.

(B) qRT-PCR results showing mRNA level of *Plin2*, *Lpin1*, *GPAT1* in iPSC-neurons.

Neuron media induced LD accumulation and microglial activation in BV2 cells.

In the *in vitro* model, we have previously demonstrated that LDs can accumulate in Tau^{V337M} iPSC -neuron as a response to Tau pathology. However, it's hard to explain the LD accumulation in microglia we found *in vivo*. Thus, we employed the media transfer experiment to treat BV2 cells, a widely used mouse microglial cell line , with neuronal conditioned media (NCM). After 24-hour treatment of NCM from WT iPSC-neurons (NCM^{WTC11}) and Tau^{V337M} iPSC-neurons (NCM^{V337M}), SRS imaging showed that NCM^{V337M} treatment induced LD

accumulation in BV2 cells (Fig. 5A). We also measured the *Plin2*, *Lpin1* and *GPAT1* gene expression level by qRT-PCR and found that all these three genes were upregulated after NCM^{V337M} treatment compared with NCM^{WTC11} treatment (Fig. 5B). To determine the inflammatory status of the LD-accumulating microglia, we measured the expression level of genes from the pro-inflammatory (M1) and anti-inflammatory (M2) pathway. TNFα and IL1αfrom the pro-inflammatory pathway were upregulated in NCM^{V337M} treated BV2 cells compared to NCM^{WTC11} treated BV2 cells, while TGFβ and IL4 from the anti-inflammatory pathway were unchanged (Fig. 5C), indicating BV2 cells were under proinflammatory status.

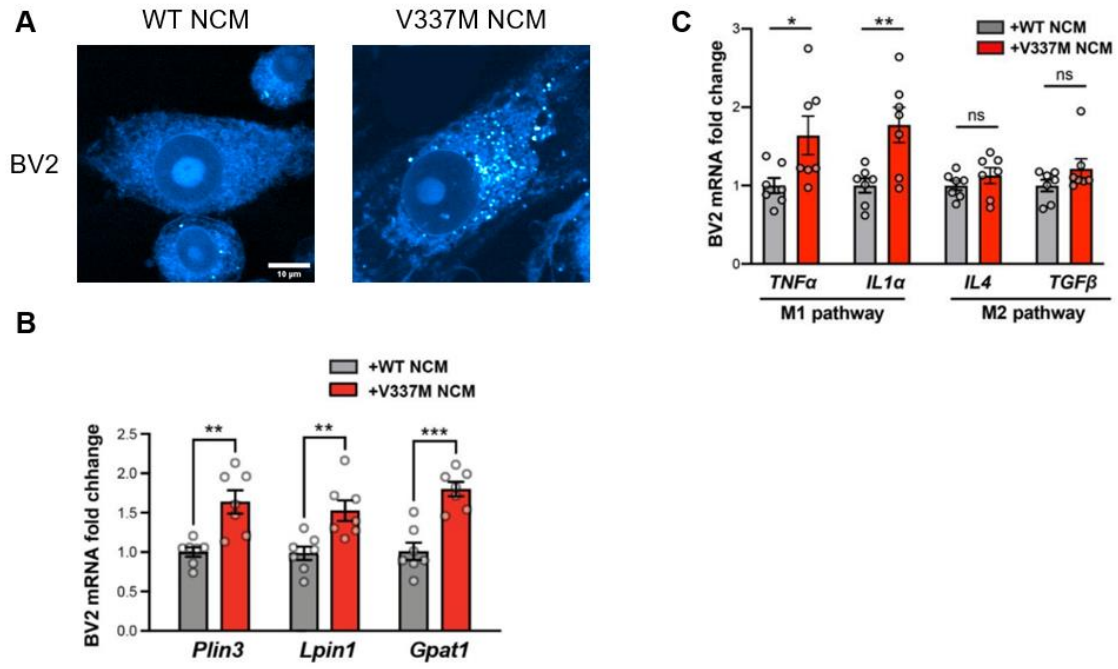


Figure 5. V337M neuron induced LD accumulation and proinflammatory response in BV2 cells.

(A) SRS images showing LDs accumulated in BV2 cells after V337M NCM treatment compared to WT NCMtreatment.

(B) qRT-PCR results showing mRNA level of *Lpin1, GPAT1* and *Plin*3 in BV2 cells after neuronal media treatment.

(C) qRT-PCR results showing mRNA level of M1/M2 pathway genes in BV2 cells after neuronal media treatment.

AMPK activity was impaired in Tau^{V337M} iPSC-Neurons.

AMPK is a very important kinase that plays a crucial role in regulating cell energy homeostasis [ref]. The activation of AMPK promotes catabolic activities and inhibit anabolic activities. Thus, we measured AMPK activity in WT and Tau^{V337M} iPSC-neurons by Western blot, and founda reduced level of pAMPK(T172), a marker for AMPK activity(Fig.6A, B). Western blot results also showed that pACC/ACC levels were reduced, confirming downregulation of AMPK activity since AMPK could inhibit ACC function by phosphorylation (Fig.6A, C).

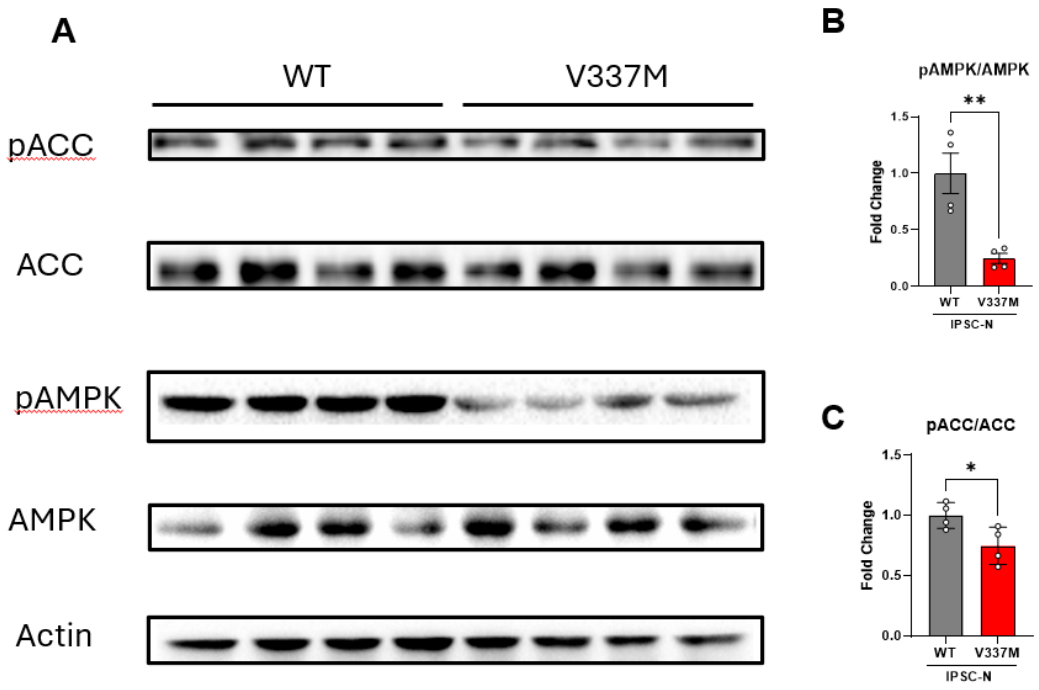


Figure 6. AMPK activity is impaired in V337M iPSC-neurons.

(A) Immunoblot images showing AMPK activity by measuring the abundance of AMPK and p-AMPK, ACC and p-ACC.

(B) Quantification of AMPK activity by normalizing p-AMPK to AMPK.

(C) Quantification of ACC activity by normalizing p-ACC to ACC.

Activation of Neuronal AMPK reduced LD accumulation in iPSC-neurons and subsequently in BV2 cells.

Since neuronal media could induce LD accumulation in BV2 cells and AMPK activity were impaired in neurons, we next asked if LD accumulation can be reduced when AMPK were activated in neurons. Thus, we used media transfer experiment again with pre-treatment of AICAR, an AMP mimic that can used as AMPK activator, in Tau^{V337M} iPSC- neurons. SRS imaging showed that LD accumulation was reduced significantly in BV2 cells after AICAR pretreatment for 72 hours (Fig. 7A). qRT-PCR showed that *Plin3*, *Lpin1* and *GPAT1* expression levels were downregulated in AICAR pre-treated NCM^{V337M} group (Fig. 7B), consistent with reduced LD accumulation in BV2 cells. We previously showed that BV2 cells could be activated by V337M NCM treatment. AICAR pre-treated Tau^{V337M} NCM induced significantly less TNFα and IL1α gene expressionin BV2 cells, as shown by qRT-PCR (Fig.7C).

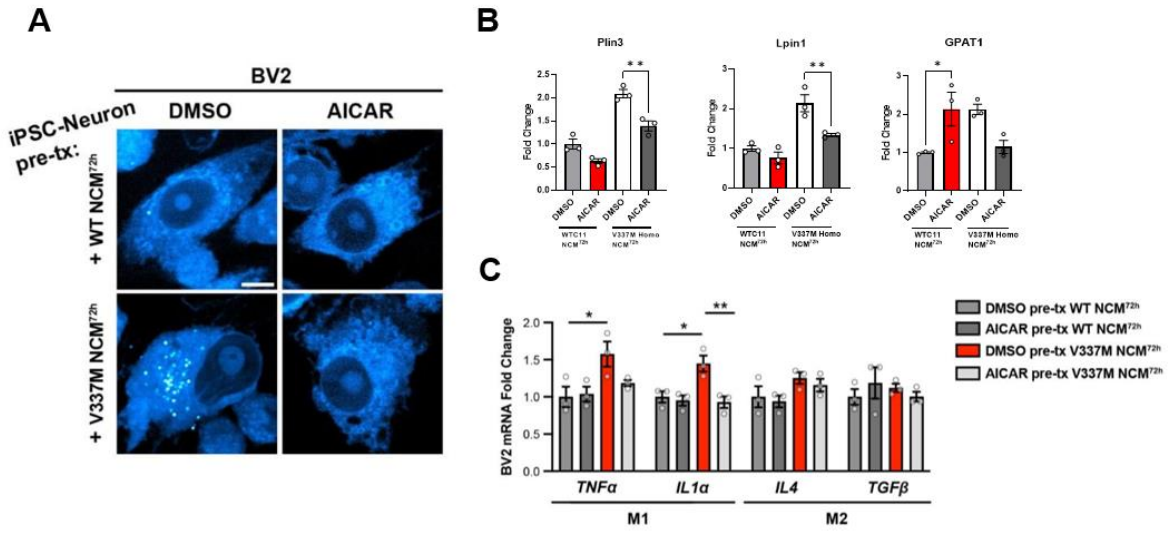


Figure 7. Neuronal AMPK activation reduced LD accumulation in BV2 cells.

(A) SRS images showing LD accumulation in BV2 cells after neuronal media treatment, with and without AICAR pretreatment of the iPSC-neurons

(B) qRT-PCR results showing mRNA levels of *Plin3, Lpin1* and *GPAT1* of BV2 cells after neuronal media treatment.

(C) qRT-PCR results showing mRNA levels of M1/M2 pathway genes of BV2 cells after neuronal media treatment.

AMPK dysfunction in Tau^{wt}-transgenic *Drosophila*

In the *in vitro* experiment, we have shown that AMPK activity was impaired in Tauopathy neurons. However, we wanted to validate if AMPK activity impairment is consistent in *in vivo* models. Thus, we tested AMPK activity in *Drosophila* model under starvation stress. Western blot showed that AMPK was activated in WT fly brain after 24-hour starvation, as shown by pAMPK level (Fig. 8A, B). However, in Tau-transgenic fly brains, AMPK activity has no significant change after 24-hour starvation (Fig. 8A,B),suggesting Tau flies lose their ability to response to starvation by activate AMPK.

Overexpression of AMPK in Drosophila neuron partially rescues the starvation response phenotype.

Since AMPK activation with AICAR in media transfer experiment demonstrated that it could reduce LD accumulation in microglial cells, we next asked if overexpression of AMPK in neurons could benefit Tau flies. Starvation lifespan assay was performed to determine flies' ability to response to starvation. AMPK.DN (dominant negative, K57D mutant) overexpression was used as negative control since AMPK.DN cannot be activated [ref]. The lifespan curve demonstrated that Tau flies have a significantly shortened lifespan under starvation compared to WT flies. Notably, overexpression of AMPK partially rescued the survival probability (Fig. 8C), potentially because AMPK overexpression contributes to the utilization of energy storage under the stress of starvation. However, since Tau flies do not have as much stored energy as WT flies, AMPK overexpression could not completely rescue the starvation lifespan.

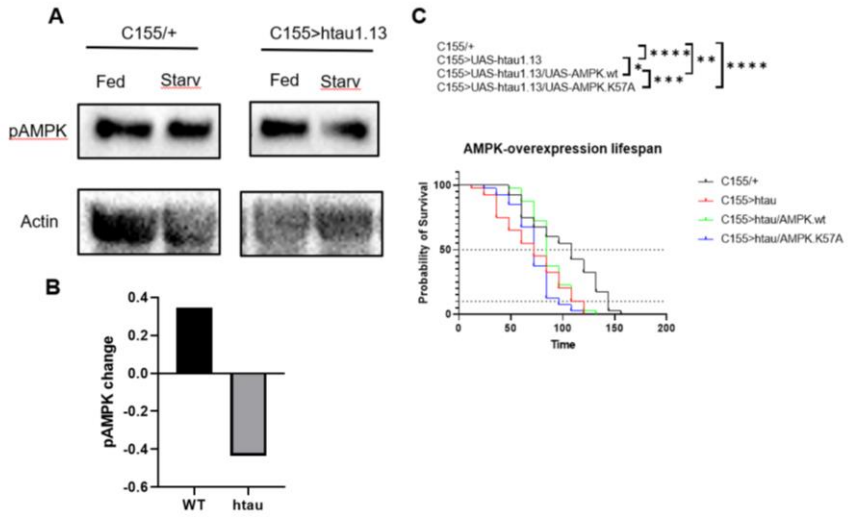


Figure 8. AMPK activity is impaired in hTau flies, which is partially rescued by overexpression of AMPK in neurons.

(A) Immunoblotting images showing p-AMPK level change under starvation in 5-day-old hTau flies and WT flies with quantification in (B).

(C) The lifespan curve of hTau and WT flies with or without AMPK overexpression under starvation. n=40 per group. *, $p < 0.05$; ***, $p < 0.001$, ****, $p < 0.0001$, ns, not significant, by Log-Rank analysis.

AMPK KO in mice hippocampus could upregulate LD related gene expression level and induce neuroinflammation in young PS19 mice.

Previous experiment demonstrated that AMPK activity impairment in tauopathy models could leads to LD accumulation and difficulties in utilizing energy storage. We next asked if inhibition of neuronal AMPK in mouse hippocampus could trigger early onset of LD accumulation and Tau pathology in PS19 mice at 3 months old. AAV-Cre or AAV-mCherry (control) were stereotaxically injected into each side of the hippocampus of *P301ShTau/prkaa1/2*$^{F/F}$ mice. SRS imaging showed significant LD accumulation in the Cre-injected hemi-hippocampus (Fig. 9C). qRT-PCR showed that *Plin3, Plin1* and *GPAT1* gene expression levels were upregulated in the Cre injection side compared to the mCherry injection side (Fig.9A), consistent with LD accumulation with AMPK depletion. Besides LD associated

gene expression level change, we also observed neuroinflammation phenotype in the Cre injected side since TGFβ and IL-4 mRNA expression levels were down-regulated in the Cre injected side while TNFα and IL-1a mRNA expression levels didn't show significant change (Fig. 9B). We also validated Cre injection induced neuroinflammation by staining with several different activated microglia markers including CD68, Clec7a and PY12RY. IHC staining results demonstrated that all these activated microglia markers were upregulated in the Cre injected side, suggesting neuronal AMPK depletion could induce microglia activation (Fig. 9C, D, E).

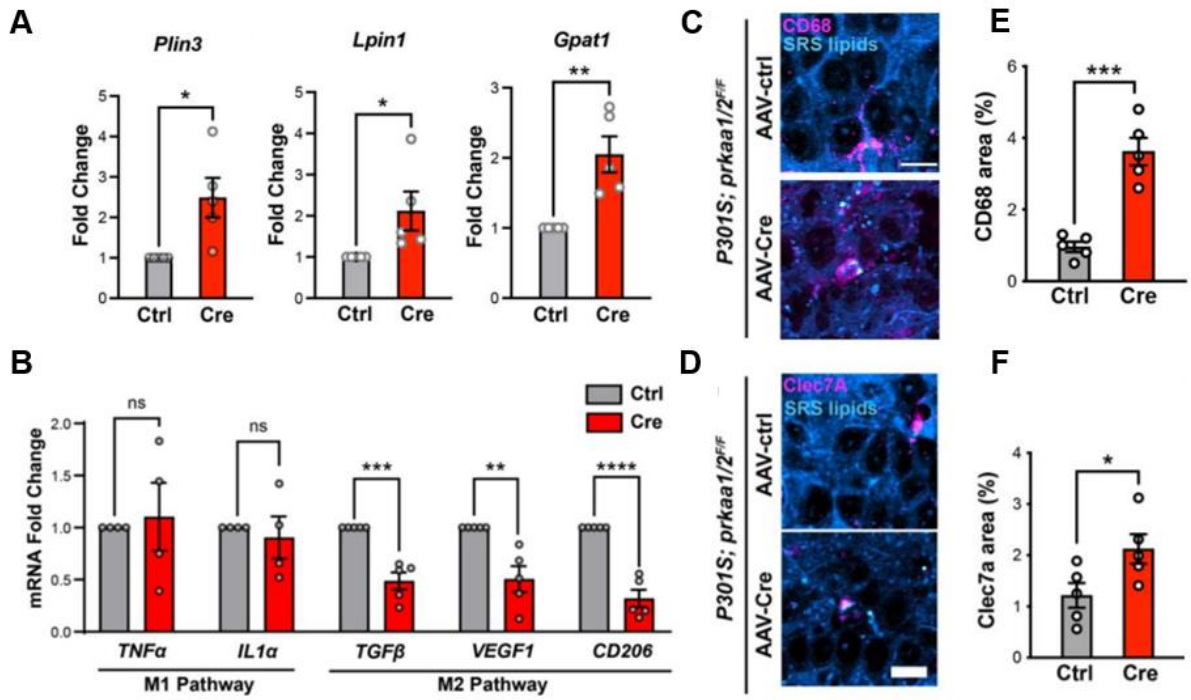


Figure 9. Neuronal AMPK depletion in hippocampus induced LD accumulation and inflammatory response in 3-month-old PS19 mice.

(A) qRT-PCR results showing mRNA level of *Plin3, Lpin1* and *GPAT1* in 3-month-old P301ShTau/PRKAA1/2$^{F/F}$ mice hippocampus. N=4.

(B) qRT-PCR results showing mRNA level of M1/M2 pathway genes in 3-month-old P301ShTau/PRKAA1/2$^{F/F}$ mice hippocampus. N=4.

(C) Immunostaining co-registered with SRS images showing CD68 in P301ShTau/PRKAA1/2$^{F/F}$ mice hippocampus with quantification in (E).

(D) Immunostaining co-registered with SRS images showing Clec7A in P301ShTau/PRKAA1/2$^{F/F}$ mice hippocampus with quantification in (F).

DISCUSSION

Disrupted glucose metabolism and over-activation of lipid metabolism in Tau-mediated neurodegenerative diseases such as AD plays an important role in contributing to the disease progression. However, the underlying mechanism remains unclear. In this project, we demonstrated a highly consistent LD accumulation phenotype across three tauopathy models *in vitro* and *in vivo*, correlated with Tau pathology. By manipulating AMPK signaling pathway,

we established neuronal AMPK as a LD accumulation modulator in the brain. By employing the SRS-imaging technique, we provide direct evidence of endogenous lipid distribution in mouse and fly brains.

RNA-seq data found a variety of lipid metabolism associated genes that have been identified as risk factors of Alzheimer's Disease, including *APOE4, TREM2, ABCA1/7* and *SREBF2* (Picard et al., 2018). In this study, we confirmed the upregulation of genes involved in lipid metabolism or LD synthesis in the context of tauopathy, which is correlated with increased lipid burden. Previous research demonstrated that Tau deposition is tightly correlated with impaired glucose metabolism in AD brain by PET imaging (Neth et al., 2017). The shift from glucose metabolism to lipid metabolism to fulfill the energy needs results in increased free fatty acids (FFA) and lipid peroxidation (Bradley Whitman et al., 2015). It's been demonstrated that oxidative stress can be induced by overabundant lipid peroxidation, which contributes to neuronal damage and neuroinflammation (Liu et al., 2017). We confirmed this observation by staining the microglia activation marker CD68 and measuring the pro-inflammatory pathway gene expression level change in Tauopathy brains.

Notably, LD accumulation in tauopathy brain mainly appears in microglia even though Tau pathology is neuron specific. Previous research reported that neuronal stress could cause the transport of lipid to astrocyte as a protective mechanism (Liu et al., 2017). Our media transfer experiment confirmed the observation. However, even though we demonstrated LD accumulation in microglia as consequences of neuronal stress, the mechanism of this neuron-glia connection is still unclear since we don't have direct evidence showing which molecule or signaling pathway are involved in lipid export from neurons that induced LD accumulation.

The previously stated risk factors such as APOE, LDLR, ABCA1 and Trem2 might be involved in the lipid transportation from neuron to microglia and LD synthesis in microglia (Loving et al., 2020). Meanwhile, neuroinflammation, which is found correlated with LD accumulation, is known to contribute to neurodegeneration. Thus, this study revealed that tauopathy neurons induced LD accumulation in microglia, which caused inflammatory response that in tern worsened the neuronal damage.

AMPK plays a role as master regulator in maintaining cellular energy state in the brain. It's been shown that AMPK expression level/activity were impaired in tauopathy (Culmsee wt al., 2001). Recent studies suggested the neuroprotective role of AMPK signaling pathways in the brain since AMPK functions in regulating Akt-GSK3b and mTOR autophagy pathways to reduce Tau phosphorylation (Kim et al., 2015). However, in this study, we identified AMPK's effect on Tauopathy in regulating LD biogenesis by inhibiting lipogenesis via Lpin1 and GPAT1. This is consistent with previous findings that AMPK regulates these two genes in hepatocytes. Moreover, AMPK could stimulate LD clearance through chaperone mediated autophagy (CMA) with Plin2/3 protein serving as CMA substrate (Kaushik et al., 2015).

In summary, our study explored the LD accumulation phenotype in tauopathy brain and investigated the role of AMPK signaling in this process.

CHAPTER III. BETA-HYDROXYBUTYRATE AMELIORATES TAUOPATHY BY ITS SIGNALING ACTIVITY

INTRODUCTION

Ketogenic diet (KD) is a diet structure that consumes high portions of fat and low or none of carbohydrate. First developed as a therapeutic intervention for epilepsy, KD is designed to induce ketosis, a metabolic state in which the body produces ketone bodies as an alternative energy resource. This metabolic shift could aid in weight loss and provides therapeutic benefits in various disease conditions including neurological diseases (Rusek et al., 2019). In the past few decades, promising evidence shows that KD could offer neuroprotective benefits and mitigate the cognitive decline in AD, especially in reducing amyloid ß plaque accumulation in AD (Rusek et al., 2019). However, long-term use of KD can lead to severe side effects including hypoglycemia, GI issues, dehydration, and nutritional deficiencies (Duchowny et al., 2005). Thus, it's important to identify the key molecule mediating the beneficial effects of KD.

ß-hydroxybutyrate (BHB) is the main ketone body produced through KD. BHB can serve as both an energy source and a signaling molecule (Qi et al., 2022). It's important to investigate whether BHB is beneficial to Tauopathy and if so, which aspect of BHB's activity plays the dominant role. To better understand BHB's effect on tauopathy brain, two isoforms of BHB, R-BHB and S-BHB, were used in this study. R-BHB could be metabolized in ß-oxidation to generate acetyl-CoA and ATP, and S-BHB preserved signaling activity of the BHB molecule while it cannot be metabolized through normal metabolic activities.

Our recent work has demonstrated that metabolic stress induces LD accumulation in tauopathy brain, and it's correlated with disease progression. Notably, BHB has been shown to

influence the lipid accumulation in adipose tissue (Lee et al., 2020). By targeting these molecular components, BHB exerts fine-tuned control over LD metabolism, orchestrating the balance between lipid storage and utilization in response to metabolic demands.

In this study, we investigated the effects of BHB in modulating LD accumulation in tauopathy using both tauopathy flies and BV2 microglia cell line. Combing genetic and pharmacological approaches *in vivo* and *in vitro*, we aimed to elucidate the mechanism underlying the effects of BHB supplementation, focusing on LD accumulation phenotype. Our finding suggests that BHB ameliorates tauopathy primarily through its signaling activity and provided evidence for BHB supplementation as a therapeutic intervention strategy to treat tauopathy.

MATERIAL AND METHODS

Drosophila Model

Drosophila lines: elav^{C155}-GAL4 and UAS-hTau1.13 was used in this study and originally obtained from BDSC. They have maintained on the standard diets for several generations and then crossed to produce Tau-overexpression Drosophila.

Lifespan Assay

C155>UAS-htau1.13 flies were collected after setting the cross. Flies were separated by sex, genotype and diet. For each condition/group, 20 flies were collected into one vial and there were 5 vials for each condition, which makes a total of 100 flies in each group. Day 0 was defined by the day that new adult flies were hatched. New flies were transferred to different diets on Day 1-2. The number of deaths in each vial were checked every two days.

Climbing Assay

60 flies from same sex, genotype and diet were collected and separated into 4 vials. All the flies were maintained on the desired diet starting from Day 1 after they hatched. Climbing assays were performed every 7 days starting from Day 7. Flies were transferred to an empty vial with standard diet at the bottom of the vial at room temperature. The measurement vial was divided into 6 regions representing climbing score. Each group of flies has a 15 second testing period starting from tapping them down to the surface of the food and starts climbing. Then they were allowed to rest for 1 min. 3 technical replicates were performed and the score from this group was determined by the average score of 3 technical replicates.

BV2 cell culture

WT BV2 cells were thawed and maintained in T25 cell culture flask with 10 ml of RPMI1640 +10% fetal bovine serum (FBS)+1%Penicillin/Streptomycin media in cell culture incubator with 5% CO_2 at 37℃. Subcultures were performed every 3 days or when the BV2 cells covered more than 90% of the cell culture flask bottom. 2 ml 0.25% Trypsin-EDTA was added into the cell culture flask after removing the old media to detach the BV2 cells for 3 mins in 37℃ incubator. Then, 4 ml fresh media were added into the cell culture flask to end Trypsin-EDTA digestion. BV2 cells were suspended and spun down at 1000 rpm for 5 mins and the cell pellet were re-suspended in 3 ml fresh culture media. 1 ml of media containing the re-suspended cell was added to the new cell culture flask with 9 ml of fresh media and incubate at 37℃.

Drug treatment experiment

BV2 cells at Passage 5-6 were seeded on coverslips in 24-well-plate at 100k cells per well with 600 ul RPMI1640 +10% fetal bovine serum (FBS)+1%Penicillin/Streptomycin media. 24 hours after seeding, 1 mM lipopolysaccharide (LPS) were added into the desired wells for 24 hours. Then, media containing LPS was removed and replaced with 5 mM NaCl/Na-R-BHB/Na-S-BHB and treat for 12 hours.

Media transfer experiment

BV2 cells at Passage 5-6 were seeded on coverslips in 24-well-plate at 100k cells per well with 600 ul RPMI1640 +10% fetal bovine serum (FBS)+1%Penicillin/Streptomycin media. 24 hours after seeding, 300 ul media were replaced by Neuronal conditioned media from WT or Tau^{V337M} iPSC-neuronal culture for 72 h. Then, NCM was removed and replaced with 5 mM NaCl/Na-R-BHB/Na-S-BHB and treat for 12 hours.

BODIPY staining

Coverslips with seeded cells were fixed in 4% PFA for 30 minutes at room temperature and then transferred to 1xPBS to wash off the rest PFA. Then, cells were treated with 200 ul 0.1%PBS-T solution for 30 minutes and stained with BODIPY for 15 minutes. After removed BODIPY was removed, the cells were washed with 1xPBS for 3 times and 10 minutes for each wash. Then, the cells stayed in 1xPBS for imaging purposes.

RESULTS

S-BHB supplementation partially rescues lifespan and motor function of Tauopathy flies.

Previous research suggested that KD is beneficial to AD and BHB is the main product from KD. Thus, we asked if BHB supplementation could ameliorate tauopathy. Utilizing a Tau transgenic fly model which overexpresses human Tau protein in neurons and exhibits severely shortened lifespan with motor function deficit, we examined BHB's effect by supplementing fly standard diet with 2 mM S- or R-BHB. We first determined the effects of BHB supplementation in tauopathy fly model for their lifespan and climbing ability. Tau flies exhibited significantly shortened health span (50% survival probability) and lifespan (10% survival probability), as expected (Fig.10A, B). Supplementation with S-BHB, but not R-BHB, significantly extended lifespan in male Tau flies (Fig.10A). Interestingly, neither R-BHB or S-BHB supplementation significantly improved lifespan or healthspan of female Tau flies (Fig.10B), suggesting sex difference in BHB response.

Next, we performed climbing assay in Tau flies supplemented with S- or R-BHB starting from 7 days old to 35 days old. Tau flies on the control diet exhibited a lower climbing score, compared to WT flies (Fig.10C). S-BHB supplementation partially rescued the motor function in male Tau flies up to 21 days old. The effect of S-BHB diminished in 35-day-old flies (Fig.10C). R-BHB supplementation had a minimal effect, presumably due to its rapid metabolism to generate energy and consequently loss of it signaling activity. Both S-BHB and R-BHB supplementation in female Tau flies showed improved climbing score compared with normal diet group (Fig.10D).

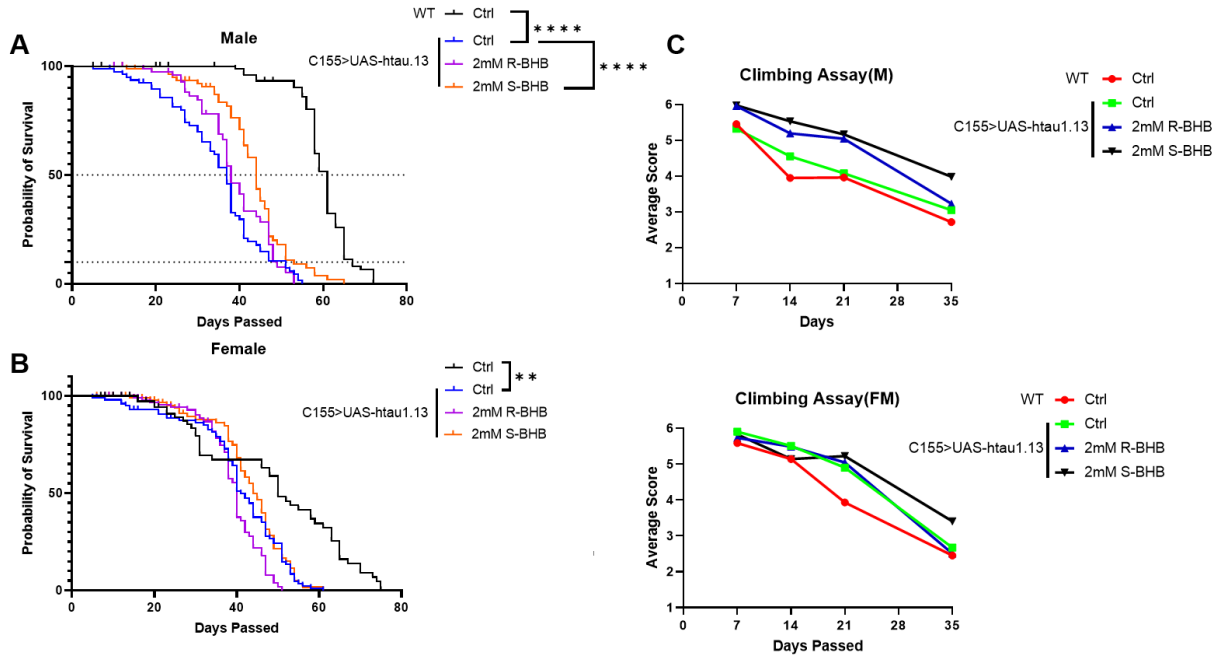


Figure 10. S-BHB supplementation partially extends lifespan and motor deficit of tauopathy flies

(A) The lifespan curve of male WT and hTau flies on Ctrl, 2 mM R-BHB supplemented, and 2 mM S-BHB supplemented diet. N=100 per group. *, $p < 0.05$; ***, $p < 0.001$, ****, $p < 0.0001$, ns, not significant, by Log-Rank analysis.

(B) The lifespan curve of female WT and hTau flies on Ctrl, 2 mM R-BHB supplemented, and 2 mM S-BHB supplemented diet. N=100 per group. *, $p < 0.05$; ***, $p < 0.001$, ****, $p < 0.0001$, ns, not significant, by Log-Rank analysis.

(C) Climbing assay result on WT and hTau flies on Ctrl, 2 mM R-BHB supplemented, and 2 mM S-BHB supplemented diet. N=50 per group.

NCM- and LPS-induced LD accumulation in BV2 cells could be reduced by S-BHB treatment.

In our previous work, we reported LD accumulation in activated microglia in response to NCM^{V337M} treatment. Here, we asked if BHB supplementation could reduce LD accumulation in microglia under inflammatory status. LPS has been used to induce inflammatory response in BV2 cells. We treated BV2 cells with 1 uM LPS for 24 hours, followed by 5 mM R-/S-BHB treatment for 12 hours. Immunostaining showed prominent LD

accumulation in BV2 cells after LPS treatment, as expected. Remarkably, S-BHB treatment significantly reduces LD accumulation, while R-BHB was less effective (Fig.11A,C) We next investigated whether S-BHB can also reduce LD accumulation and inflammatory response after NCM^{V337M} treatment. Similar to what we observed with LPS treatment, S-BHB, but not R-BHB, reduced LD accumulation in BV2 cells treated with NCM (Fig.11B,D). Together, these results suggest that the signaling activity of BHB is involved in regulating LD synthesis or lipolysis pathway.

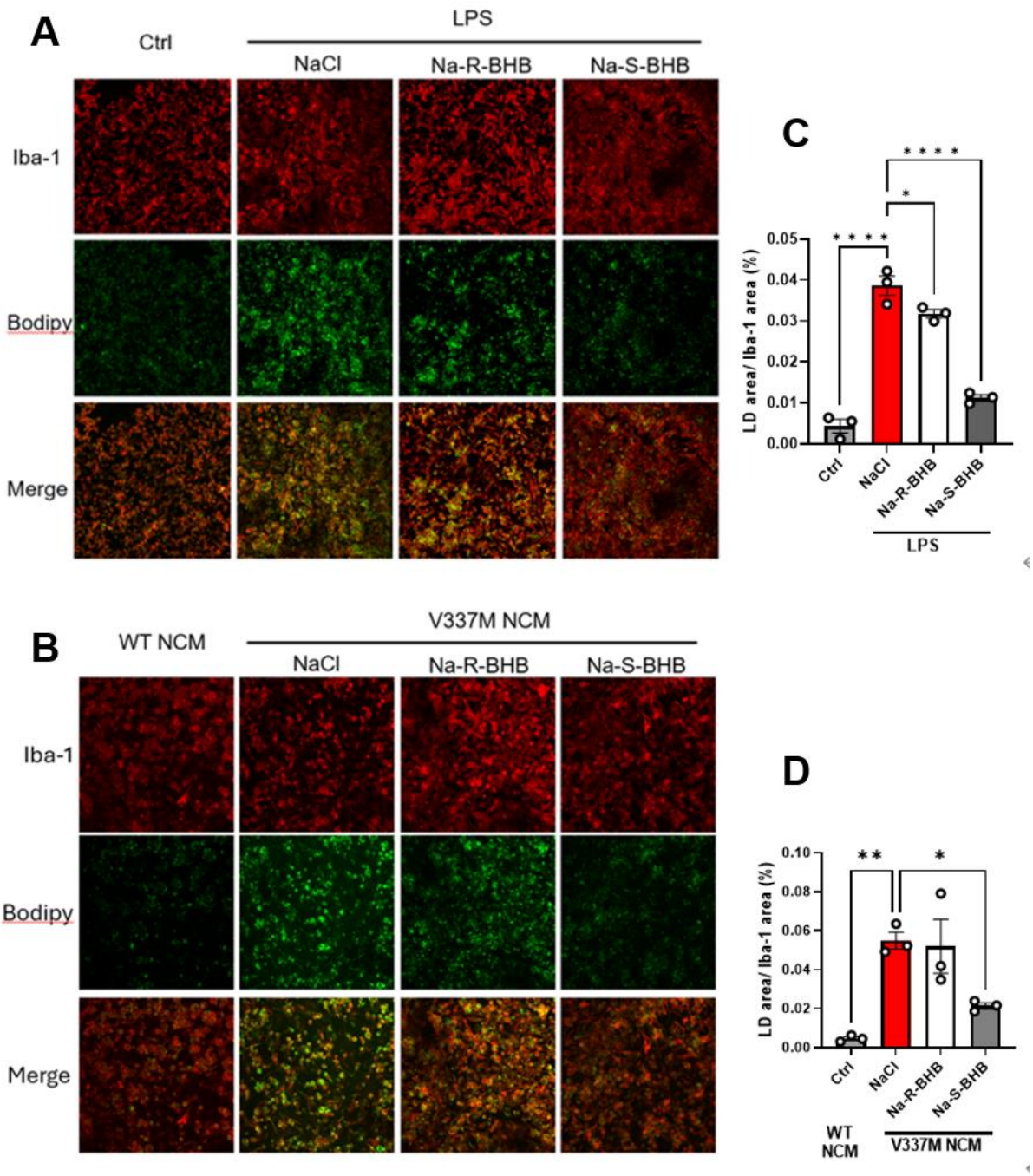


Figure 11. S-BHB supplementation reduced LD accumulation in BV2 cells treated with LPS or NCM^{V337M}.

(A) Immunostaining images of LD distribution in LPS treated BV2 cells with or without BHB treatment. Quantification in (C)

(B) Immunostaining images of LD distribution in NCM treated BV2 cells with or without BHB treatment. Quantification in (D)

DISCUSSION

BHB supplementation provides another point of view in ameliorating the Tau pathology. Previous research demonstrated that ketogenic diet is contributing to improving the cognitive ability and motor function of AD patients. In this study, BHB, which is the main product from ketogenic diet, was used in tauopathy flies and microglia cultures to determine the effect. Our study revealed that S-BHB could significantly improve the behavioral phenotype of Tauopathy flies, suggesting that BHB benefits Tauopathy animals mostly through its signaling activity. However, further research is needed to determine which signaling pathways mediate the effects of BHB.

We have shown earlier that LD accumulation phenotype is closely related to tauopathy. Here, we demonstrated that BHB supplementation could reduce LD accumulation in microglial cells, suggesting BHB may be involved in regulating LD metabolism to ameliorate tauopathy phenotypes. Previous research demonstrated that BHB could interact with LD associated proteins to regulate LD metabolism in adipose tissues (Wang et al., 2019). In this study, we used media transfer experiment to provide direct evidence that BHB reduces LD accumulation in microglia cells. However, it's still worth investigating whether BHB also functions in other types of cells, for instance, neurons in the setting of tauopathies.

In summary, our study provides *in vivo* evidence of the protective effects of BHB supplementation in tauopathy. Meanwhile, we elucidated the potential mechanism underlying how BHB regulates LD accumulation in microglia cells, thus contributing to the neuroprotective effect of BHB. Our results provide a foundation for future mechanistic and translational studies on using ketone body as an intervention approach to treat AD and other

tauopathies.

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